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Full Length Research Paper

Optimization of cholesterol oxidase production by *Brevibacterium* sp. employing response surface methodology

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An ultrasound-assisted emulsification as a pretreatment for cholesterol oxidase production by submerge fermentation using *Brevibacterium* sp. in a batch system was studied. Medium improvement for the production employing response surface methodology (RSM) was optimized in this paper. The concentration of Tween-80, cholesterol and the time of ultrasonic pretreatment medium were further optimized by RSM. Results from RSM showed that the initial concentration of cholesterol, Tween-80 and pretreatment time exerted a significant effect on cholesterol oxidase production, but these factors did not exert a significant effect on cell growth. The improved medium consisted of 4.076 g/L cholesterol, 0.2932‰ (v/v) Tween-80, 22.361 (min) treatment time, and cholesterol oxidase production reached 1.483 U/ml after 36 h culture, which was 83.57% greater than the control medium.

Key words: Ultrasonic, cholesterol oxidase, response surface methodology.

INTRODUCTION

Ultrasonic is a sound wave with frequency beyond the normal hearing range of humans (>15 to 20 KHz). When ultrasonic wave propagates in a liquid medium, it can produce cavitation and acoustic streaming. This cavitation generates powerful shear forces, while the acoustic streaming increases the convection of solution (Guo et al., 2010; Suslick et al., 1999; Khanal et al., 2007).

Effect of ultrasonic on reaction process has aroused strong interest and high attention with the popularity and development of ultrasonic equipment in recent years. Studies showed that a suitable intensity of ultrasonic irradiates biological reaction medium can enhance efficiency of emulsification, increase the permeability and selective of cell membranes, promote the secretion of enzymes and enhance cell metabolism and thus shorten the reaction time, improve product quality and yields.

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There are many reports about this aspect of research, such as the stimulating fermentative activities of bifidobacteria in milk by high intensity ultrasound (Thi My Phuc et al., 2009). Their results showed that the probiotic bacteria cells were ruptured by ultrasound and released intracellular enzyme β-galactosidase that promoted the hydrolysis of lactose and trans-galactosylation, and subsequently enhanced the growth of the remaining bacterial cells in inoculated milk during fermentation. The lower the concentration of lactose, the higher the amount of oligosaccharides (degree of polymerization = 3) found in the fermented milk with ultrasound treatment. Chang et al. (2007) also studied the enhancement of *Bacillus thuringiensis* (*Bt*) production from sewage sludge with alkaline and ultrasonic pretreatments. Suitable pretreatment conditions were optimized with 5 g/L sodium hydroxide for alkaline treatment and 1.2×10^5 kJ/kg of total solid for ultrasonic treatment. Fermentations of raw and pretreated sludge for biopesticides were carried out in a bench scale fermentor. These authors observed that both pretreatments were effective for *Bt* growth and

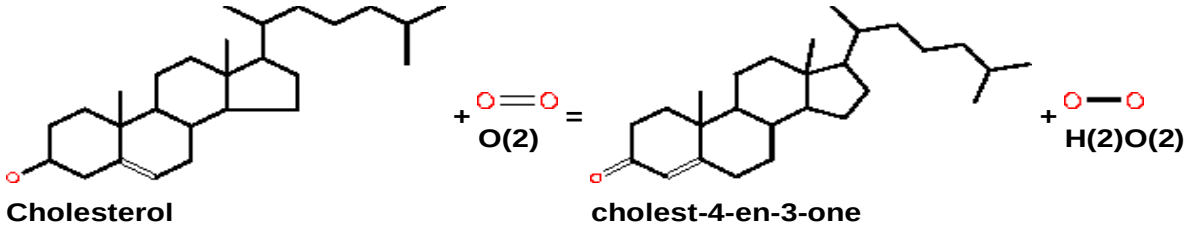

Figure 1. The oxidation and isomerization reaction of cholesterol.

metabolism. Higher viable cells and viable spores' counts, δ-endotoxin yields and entomotoxicity were achieved in the pretreated sludge. In addition, Runyan et al. (2006) used low-frequency ultrasonic to increase outer membrane permeability of microorganism. Gaikwad and Pandit (2008) carried out ultrasonic emulsification of oil and water and studied the effect of irradiation time, irradiation power and physicochemical properties of oil on the dispersed phase volume and dispersed phase droplet size.

Meanwhile, increasing *Brevibacterium* sp. with a high cholesterol oxidase (COD) yield for reducing cholesterol by means of biological technique remains an importance task for researchers. COD from *Brevibacterium sp*., a flavin adenine dinucleotide (FAD)-dependent enzyme, catalyzes the oxidation and isomerization of cholesterol into cholest-4-en-3-one and yields hydrogen peroxide (Figure 1). However, some cholesterol oxidases like those from *Burkholderia cepacia* strain ST-200, *Pseudomonas* sp. and *Chromobacterium* sp. strain DS-1 oxidize cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO) but not the cholest-4-en-3-one produced by most cholesterol oxidases (Doukyu et al., 1999, 2008, 2009). Cholesterol oxidase has two major biotechnological applications; in the determination of serum (and food) cholesterol levels and as biocatalyst providing valuable intermediates for industrial steroid drug production. This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. Moreover, COD exhibits a potent insecticide activity and can inhibit the growth and generation of Lepidoptera (Purcell et al., 1993; Cho et al., 1995). It has also been reported that COD can degrade food cholesterol efficiently and economically, especially egg yolk cholesterol (Aihara and Watanabe, 1988; Speroulla et al., 1994). COD from different micro-organisms has different physicochemical properties and substrate specificity. *Brevibacterium* sp. isolated previously from soil and subjected to genetic improvement, showed extracellular COD production with cholesterol as the inducer and supplementary carbon source (Wenming, 2000).

Response surface methodology (RSM) is an efficient strategy to determine the optimum conditions for a multi-variable system with a rational number of experiments. In addition, it is less laborious and time-consuming than other approaches that are applied to optimize a process. It has already been successfully applied in medium optimization of many bioprocesses (Kamran et al., 2010; Lucio et al., 2011; Lotfy et al., 2007; Mutalik et al., 2008). The current paper describes the effect of cholesterol dispersion-improving factors on COD production and optimal media design of the factors.

MATERIALS AND METHODS

Microorganism and culture conditions

Brevibacterium sp. DGCDC-2, preserved in our laboratory was used throughout this study. Agar medium consisted of 0.3% beef extract, 0.5% NaCl, 1% peptone and 2% agar; the pH was adjusted to 7.5 with 0.1 M NaOH. Seed culture medium consisted of 0.3% beef extract, 0.5% NaCl, 1% peptone; the pH was adjusted to 7.5 with 0.1 M NaOH. The fermentation medium consists of 2% glucose, 0.15% cholesterol, 0.75% yeast extract, 0.1% NaCl, 0.01% $CaCl_2$, 0.2% CH_3COONH_4, 0.02% K_2HPO_4, 0.005% $MgSO_4.7H_2O$, 0.001% $FeSO_4.7H_2O$ and 0.05% Tween-80; pH was adjusted to 7.5 with 0.1 M NaOH.

After being autoclaved for 20 min, 150 ml aliquot of culture medium was inoculated with 5 ml activated seed culture and subjected to incubation in a 500 ml Hinton's flask at 30°C for 36 h in an orbital shaker.

Ultrasonic pretreatment medium

For dispersing cholesterol, the culture medium of *Brevibacterium* sp. was treated by ultrasonic before autoclaving. Irradiation times were 15, 20 and 25 min.

Measurement of cholesterol oxidase

Briefly, 50 µL crude enzyme were incubated with 3 ml solution A (4-amino-antipyrine, 1 mmol/L; phenol, 6 mmol/L; sodium azide, 0.2 g/L; peroxidase, 5000 U/L; potassium phosphate buffer, 25 mmol/L; pH, 7.5) and 150 µL solution B (cholesterol, 8.26 mg/ml; Triton X-100, 4.26%; isopropanol for solvent) for 5 min at 37°C, then boiled, and was measured by spectrophotometry at 500 nm. The enzyme activity was calculated as:

$$\text{Enzyme activity (U/ml)} = 0.1315A_{500} \times 3.2 \times 20 \div 5 = 1.6832 \times A_{500}$$

Design of experiments

To explore the effect of the dispersion conditions variables on the response in the region of investigation, a central composite design

Table 1. Independent variables and their coded and values.

Variable	Symbol	Coded level		
		-1	0	1
Cholesterol content (g/l)	X_1	3.5	4.0	4.5
Tween-80 content (‰, v/v)	X_2	0.2	0.3	0.4
Ultrasonic pretreatment time(min)	X_3	15	20	25

Table 2. The 3×3 factorial central composite designs and response values for medium optimization of cholesterol oxidase production.

Trial	X_1	X_2	X_3	COD production (U/ml)
1	-1	-1	0	0.8932
2	-1	0	-1	0.7251
3	-1	0	1	0.9349
4	-1	1	0	0.9573
5	0	-1	-1	1.2794
6	0	-1	1	1.3342
7	0	1	-1	1.1906
8	0	1	1	1.3876
9	1	-1	0	1.1953
10	1	0	-1	1.1633
11	1	0	1	0.9347
12	1	1	0	1.0884
13	0	0	0	1.4041
14	0	0	0	1.4583
15	0	0	0	1.4528

COD, Cholesterol oxidase.

at three levels was performed. Tween-80, cholesterol and ultrasonic pretreatment medium were selected as independent variables. The range of values and coded levels of the variables are given in Table 1. A polynomial equation was used to predict the response as a function of independent variables and their interactions. In this work, the number of independent variables was three and therefore the response for the quadratic polynomials becomes:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12} X_1 X_2 + A_{13} X_1 X_3 + A_{23} X_2 X_3 + A_{11} X_1^2 + A_{22} X_2^2 + A_{33} X_3^2$$

Where Y is the predicted response, X_1 is the cholesterol, X_2 is the Tween-80, X_3 is the ultrasonic pretreatment time, A_0 is the intercept coefficient, A_j are the linear terms, A_{ij} are the interaction terms and A_{jj} are the square terms.

Statistical analysis

Data were processed using analysis of variance (ANOVA) to obtain the interaction parameters between the process variables and response. The fit quality of polynomial model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the F-test.

RESULTS AND DISCUSSION

The experimental results on the effect of the independent variables namely Tween-80, cholesterol and ultrasonic pretreatment time on the COD production are shown in Table 2. The coefficients of the variables in the models and corresponding R^2 are shown in Table 3. The statistical analysis indicated that the proposed model was adequate and with satisfactory values of R^2. The closer the value R^2 is to unity, the better the empirical model fits the actual data. The R^2 value for COD production were 0.9917, indicating that the regression models explained the reaction well. The probability (p) values of all regression models were less than 0.001 except for browning indexes ($p < 0.05$). Table 4 clearly shows that the COD production were significantly affected by the linear ($p < 0.001$) and quadratic ($p < 0.01$) effects of cholesterol and ultrasonic pretreatment time. It was found that the linear terms of cholesterol and ultrasonic pretreatment time had a positive effect on the COD production, and showing a significantly effect in the quadratic term. The effect of interaction between cholesterol and ultrasonic pretreatment time on the COD production was negative ($p < 0.05$). Tween 80 showed no significant effect on the COD production ($p < 0.05$).

The effect of cholesterol and Tween 80 on the variation of the COD production at fixed ultrasonic pretreatment time is shown in Figure 2. It may be observed that the

Table 3. Analysis of variance for the regression model of medium optimization of cholesterol oxidase production obtained from the experimental results.

Source	Degree of freedom	Sum of square	R^2	*F*-value	Prob > F
Linear	3	0.155185	0.2095	42.110	0.0006
Quadratic	3	0.567080	0.7654	153.9	0.0000
Cross product	3	0.012454	0.0168	3.379	0.1115
Total regress	9	0.734719	0.9917	66.456	0.0001
Total error	5	0.006142			
Sum	14	0.740861			

COD, Cholesterol oxidase.

Table 4. Regression coefficients of a full second-order polynomial model for medium optimization of cholesterol oxidase production.

Source	Degree of freedom	Coefficients estimated	Standard deviation	T-value	Prob > |T|
Constants	1	1.438400	0.020235	71.084	0.0000
X1	1	0.108900	0.012392	8.788	0.0003
X2	1	-0.009775	0.012392	-0.789	0.4659
X3	1	0.086275	0.012392	6.962	0.0009
X1*X1	1	-0.381650	0.018240	-20.924	0.0000
X2*X1	1	-0.042750	0.017524	-2.439	0.0587
X2*X2	1	-0.023200	0.018240	-1.272	0.2593
X3*X1	1	0.004700	0.017524	0.268	0.7993
X3*X2	1	0.035550	0.017524	2.029	0.0983
X3*X3	1	-0.117250	0.018240	-6.428	0.0014

COD, Cholesterol oxidase.

effect of cholesterol on the yield is curvilinear in nature at constant ultrasonic pretreatment time and Tween 80. The effect of Tween 80 on the yield is non-linear at fixed cholesterol and ultrasonic pretreatment time, and the interaction with cholesterol and Tween 80 was negative ($p < 0.05$). The effect of Tween 80 and ultrasonic pretreatment time on the variation of the COD production at fixed cholesterol is shown in Figure 3. It may be observed that the effect of ultrasonic pretreatment time on the yield is curvilinear in nature at constant cholesterol and Tween 80. The effect of Tween 80 on the yield is non-linear at fixed cholesterol and ultrasonic pretreatment time. The interaction with ultrasonic pretreatment time and Tween 80 is negative ($p < 0.05$). In addition, Figure 4 shows COD production increase with Tween 80 at fixed cholesterol and ultrasonic pretreatment time. However, the effect of Tween 80 on yield is not significant.

COD from different microorganisms has different physicochemical properties and substrate specificity. The *Brevibacterium* sp. showed extracellular COD production with cholesterol as the inducer and supplementary carbon source, which suggested that the interface contact between cholesterol and the microorganism might be a key factor influencing COD production. Although methods employing the emulsifier to improve COD production were reported quantitatively, it is far from being satisfactory. Ultrasonic has a strong biological effect. The mechanism is very complex, but the major effect might be the cavitation. Cavitation is a series of dynamics courses: vibration, enlargement, shrinking, and even collapse may occur when using ultrasonic treatment. These courses happen when small air bubbles (vapor bubbles, or holes) form in liquid phase. Cavitation bubbles shrink in heat insulation condition, and even instantly collapse. In the twinkling, the super high temperature of 5000°C and several thousand atmosphere may exist near bubbles, and associating with the powerful shockwave or shooting flow, it may lead to the change of interfacial tension, and generate the stable emulsification system. Thus, microorganisms can contact fully with the substrate and promote COD production.

Conclusion

Canonical analysis, a mathematical approach to examine the over-all shape and locate the stationary point of the response surface, showed that the stationary point of the current response surface was a maximum response. At the optimum medium composition: 4.076 g/L cholesterol, 0.2932‰ (v/v) Tween-80,22.361(min) treatment time, and COD production which reached 1.483U/ml was

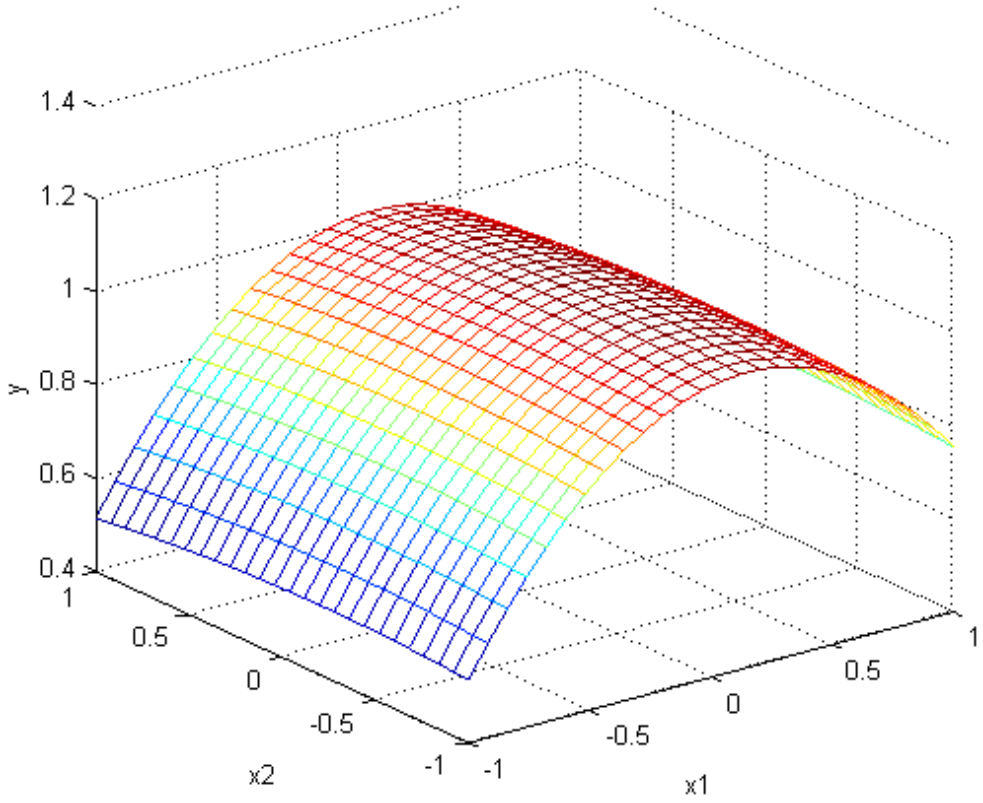

Figure 2. Effect of cholesterol and Tween 80 concentration on cholesterol oxidase production.

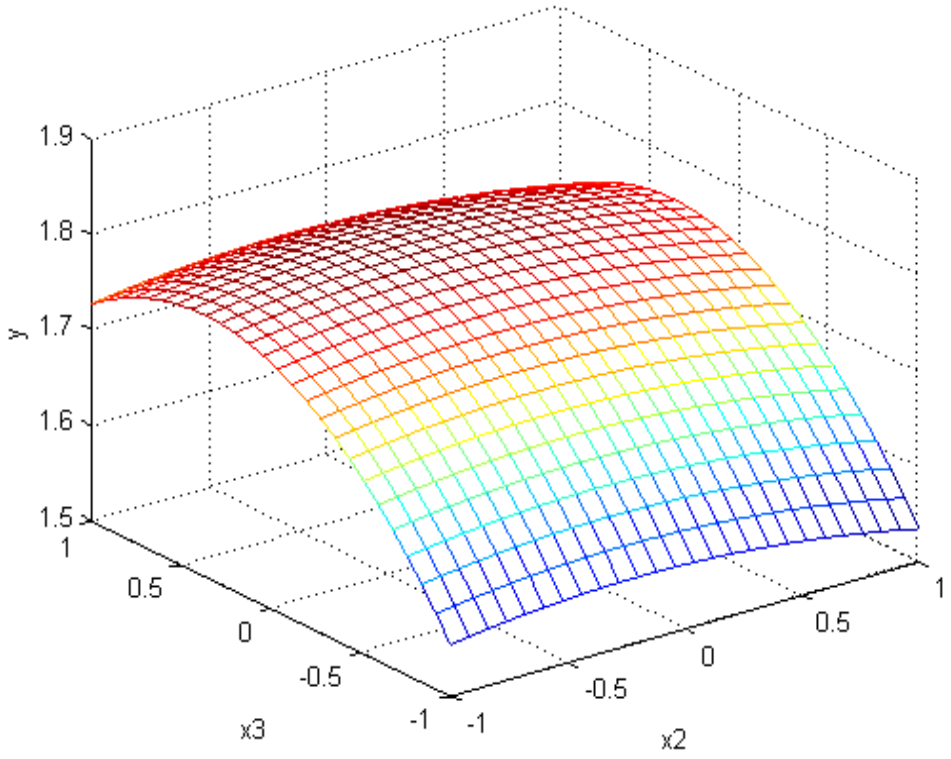

Figure 3. Effect of Tween 80 and ultrasonic pretreatment time concentration on cholesterol oxidase production.

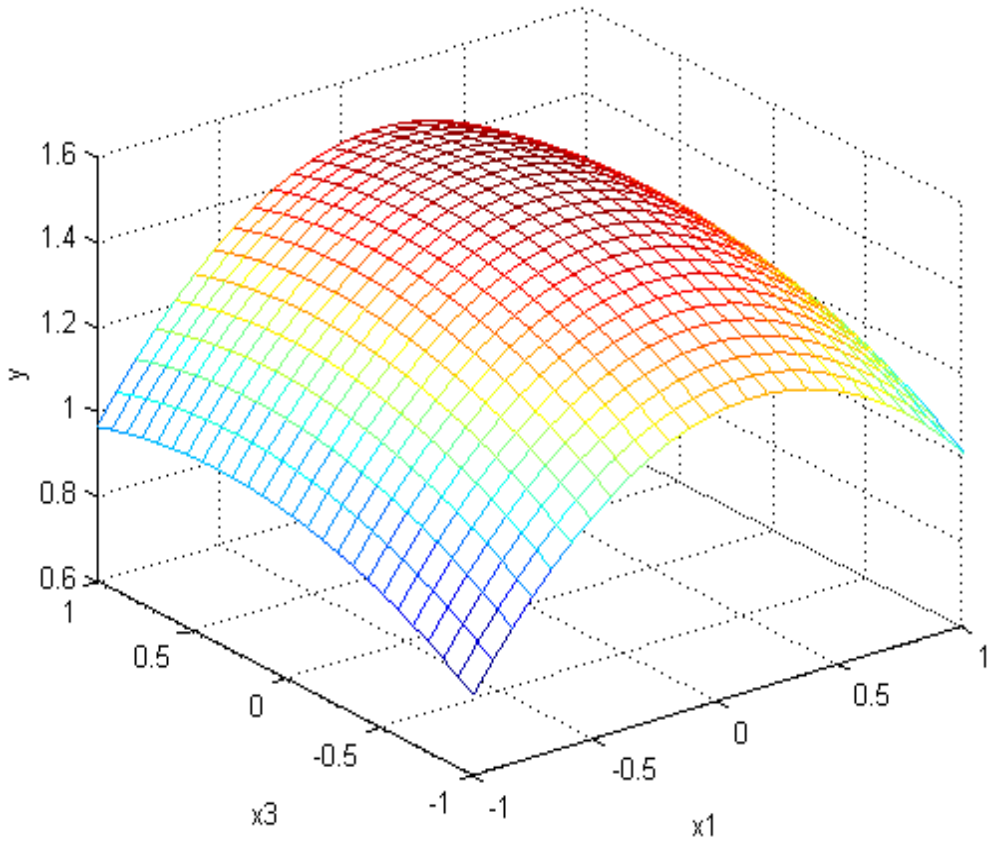

Figure 4. Effect of cholesterol and ultrasonic pretreatment time concentration on cholesterol oxidase production.

predicted. *Brevibacterium* sp. was incubated in optimized medium in 500 ml shake-flasks. Maximum COD production of 1.469 U/ml was observed, quite close to the predicted maximum response value. Hence, COD production rate could be increased 83.57% greater than the control medium when ultrasonically stimulated directly on broth.

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